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# On the recovery of Cys-containing peptides during peptide mapping by HPLC

## Tryptic peptides of Trp-tRNA synthetase of *E.coli*

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Conditions are presented for separating the major tryptic peptides of *E.coli* tryptophanyl-transfer RNA synthetase by reversed-phase liquid chromatography using a water-methanol gradient in the presence of 0.1% trifluoroacetic acid. Three of the peptides contain cysteine and are recovered in good yields if alkylated, but otherwise cannot be detected. A convenient post-digestion alkylation procedure is appropriate for use with small samples of protein which can be digested under reducing conditions. These results will be of interest for studies of the labeling of sulfhydryl groups in other proteins.

*Reversed-phase HPLC* *Tryptic peptide mapping* *Trp-tRNA synthetase* *Cys-alkylation effect*

## 1. INTRODUCTION

Tryptophanyl-tRNA synthetase from *E. coli* (EC 6.1.1.2) is an example of an enzyme whose amino acid sequence was deduced from its gene sequence [1]. Tryptic peptides representing about two-thirds of the molecule had previously been sequenced by conventional peptide methods [2]. Three Cys-containing peptides were identified by peptide mapping [3], and the fourth Cys residue was found from the gene sequence [1] to occupy position 37, only 3 residues away from Cys-40.

This report concerns a method for digesting, separating and identifying the major tryptic peptides from about 6 nmol Trp-tRNA synthetase. The effect of alkylation on the recovery of the Cys-containing peptides is discussed.

## 2. MATERIALS AND METHODS

Plasmid pCH6 and *E. coli* strain W3110 were generous gifts from Professor Charles Yanofsky. Strain W3110 was transformed with pCH6 [4], and tryptophanyl-tRNA synthetase was purified from the transformed strain by the method of Joseph and Muench [5]. Peptide mapping was carried out on a 5–10 nmol scale. The pH and ionic strength of the enzyme solution were adjusted using a small column of Bio-gel P-2 (Biorad Laboratories) immediately prior to protease digestion. 300 μl of a solution containing typically about 0.8 mg/ml Trp-tRNA synthetase in 10 mM β-mercaptoethanol and 0.1 M potassium phosphate (pH 7.0), were chromatographed on a 1-ml column of Bio-Gel P-2, equilibrated and eluted with 50 mM bicine (pH 8.0). The first 400 μl to elute from the column were discarded, and the enzyme was collected (95% recovery) in the next 300 μl. The sample was then digested with trypsin for a total of 4 h at 37°C. 2-μl aliquots (2 μg, 0.8% of weight of Trp-tRNA synthetase) of a stock solution of 1 mg/ml trypsin (Worthington, 3× crystallized) were added at 0, 1, and 2 h digestion time. Selected samples were treated post-digestion at 37°C with 15 μl of 20 mM dithiothreitol (Calbiochem) for 20 min, followed by 30 μl of 40 mM iodoacetamide (Sigma, recrystallized) for 2 h. Samples were then frozen and stored at −20°C.

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Table 1
Expected tryptic peptides from *E. coli* Trp-tRNA synthetase

| | | Sequence numbering |
|---|---|---|
| 1. | Met-Thr-Lys | (1–3) |
| 2. | Pro-Ile-Val-Phe-Ser-Gly-Ala-Gln-Pro-Ser-Gly-Glu-Leu-Thr-Ile-Gly-Asn-Tyr-Met-Gly-Ala-Leu-Arg | (4–26) |
| 3. | Gln-Trp-Val-Lys | (27–30) |
| 4. | Met-Gln-Asp-Asp-Tyr-His-Cys-Ile-Tyr-Cys-Ile-Val-Asp-Gln-His-Ala-Ile-Thr-Val-Arg | (31–50) |
| 5. | Gln-Asp-Ala-Gln-Lys | (51–55) |
| 6. | Leu-Arg-Lys | (56–58) |
| 7. | Ala-Thr-Leu-Asp-Thr-Leu-Ala-Leu-Tyr-Leu-Ala-Cys-Gly-Ile-Asp-Pro-Glu-Lys | (59–76) |
| 8. | Ser-Thr-Ile-Phe-Val-Gln-Ser-His-Val-Pro-Glu-His-Ala-Gln-Leu-Gly-Trp-Ala-Leu-Asn-Cys-Tyr-Thr-Tyr-Phe-Gly-Glu-Leu-Ser-Arg | (77–106) |
| 9. | Met-Thr-Gln-Phe-Lys | (107–111) |
| 10. | Asp-Lys | (112–113) |
| 11. | Ser-Ala-Arg | (114–116) |
| 12. | Tyr-Ala-Glu-Asn-Ile-Asn-Ala-Gly-Leu-Phe-Asp-Tyr-Pro-Val-Leu-Met-Ala-Ala-Asp-Ile-Leu-Leu-Tyr-Gln-Thr-Asn-Leu-Val-Pro-Val-Gly-Glu-Asp-Gln-Lys | (117–151) |
| 13. | Gln-His-Leu-Glu-Leu-Ser-Arg | (152–158) |
| 14. | Asp-Ile-Ala-Gln-Arg | (159–163) |
| 15. | Phe-Asn-Ala-Leu-Tyr-Gly-Glu-Ile-Phe-Lys | (164–173) |
| 16. | Val-Pro-Glu-Pro-Phe-Ile-Pro-Lys | (174–181) |
| 17. | Ser-Gly-Ala-Arg | (182–185) |
| 18. | Val-Met-Ser-Leu-Leu-Glu-Pro-Thr-Lys-Lys | (186–195) |
| 19. | Met-Ser-Lys | (196–198) |
| 20. | Ser-Asp-Asp-Asn-Arg | (199–203) |
| 21. | Asn-Asn-Val-Ile-Gly-Leu-Leu-Glu-Asp-Pro-Lys | (204–214) |
| 22. | Ser-Val-Val-Lys-Lys | (215–219) |
| 23. | Ile-Lys-Arg | (220–222) |
| 24. | Ala-Val-Thr-Asp-Ser-Asp-Glu-Pro-Pro-Val-Val-Arg | (223–234) |
| 25. | Tyr-Asp-Val-Gln-Asn-Lys | (235–240) |
| 26. | Ala-Gly-Val-Ser-Asn-Leu-Leu-Asp-Ile-Leu-Ser-Ala-Val-Thr-Gly-Gln-Ser-Ile-Pro-Glu-Leu-Glu-Lys | (241–263) |
| 27. | Gln-Phe-Glu-Gly-Lys | (264–268) |
| 28. | Met-Tyr-Gly-His-Leu-Lys | (269–274) |
| 29. | Gly-Glu-Val-Ala-Asp-Ala-Val-Ser-Gly-Met-Leu-Thr-Glu-Leu-Gln-Glu-Arg | (275–291) |
| 30. | Tyr-His-Arg | (292–294) |
| 31. | Phe-Arg | (295–296) |
| 32. | Asn-Asp-Glu-Ala-Phe-Leu-Gln-Gln-Val-Met-Lys | (297–307) |
| 33. | Asp-Gly-Ala-Glu-Lys | (308–312) |
| 34. | Ala-Ser-Ala-His-Ala-Ser-Arg | (313–319) |
| 35. | Thr-Leu-Lys | (320–322) |
| 36. | Ala-Val-Tyr-Gln-Ala-Ile-Gly-Phe-Val-Ala-Lys | (323–333) |
| 37. | Arg | (334) |

For the separation of peptides, a digested sample was removed from the freezer, centrifuged 1 min in a microfuge (Eppendorf), and chromatographed at room temperature on a 4.6 × 250 mm Brownlee Aquapore RP-300 column. The peptides resulting from each 300-$\mu$l digestion were injected onto the column and eluted using a complex 225-min gradient from 0.1% trifluoroacetic acid (Aldrich) in $H_2O$ to 0.07% trifluoroacetic acid in methanol (Baker, HPLC grade), pumped at 1.0 ml/min. The details of the gradient are illustrated in the figures.

Individual peptide fractions were hydrolyzed 20 h in constant boiling HCl, and analyzed on a microprocessor-controlled microbore amino acid analyzer designed at the University of Arkansas [6].

## 3. RESULTS AND DISCUSSION

The expected tryptic peptides based on the gene sequence for *E. coli* Trp-tRNA synthetase [1] are shown in table 1. In these experiments, we emphasized separation of the larger peptides and paid particular attention to the Cys-containing peptides, numbers 4, 7 and 8. Methanol rather than acetonitrile was chosen for the elution solvent because of the low cost and ease of handling methanol. We found that extending the time of a gradient significantly improved the resolution of peptides when using acidic methanol-water gradients at room temperature.

Fig.1 shows a 4-h gradient elution profile for tryptic peptides of unalkylated *E. coli* Trp-tRNA synthetase. Peptides 4, 7 and 8 (table 1) are notably absent from the profile. If the peptide mixture is alkylated after the proteolytic digestion but before the column chromatography, then peptides 4, 7 and 8 appear in the second half of the gradient (fig.2). The result is most dramatic for peptide 4, which is recovered in high yield. Peptide 8 elutes in moderate yield just prior to peptide 26, while peptide 7 is split between two peaks due to incomplete digestion of the bond between peptides 6 and 7 (lysine 58).

The rationale for post-digestion alkylation has to do with the ease of handling small samples. It can be difficult to remove excess alkylating agent from nmol samples of protein without undue loss of the protein itself. We found that trypsin digestion proceeded favorably under reducing conditions (10 mM mercaptoethanol), that 4 mM iodoacetamide post-digestion was sufficient to alkylate peptide 4 in high yield, and that 4 mM iodoacetamide could be injected onto the Brownlee HPLC column without interfering with the peptide mapping. Such a procedure uses a relatively low level of iodoacetamide because the alkylation takes place at the peptide stage rather than at the intact protein stage. With this method, the problem of removing a large excess of alkylating agent prior to protease digestion is circumvented.

Several factors could contribute to the apparent 'loss' of Cys-containing peptides when an alkylation step is omitted. It is possible that these

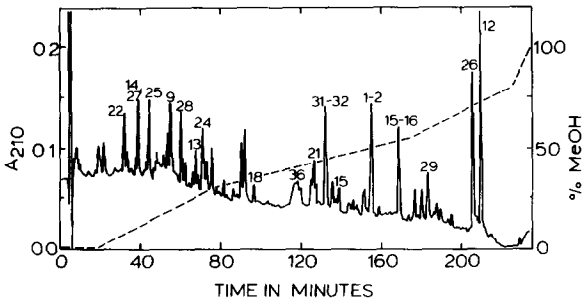


Fig.1. Peptide map of unalkylated Trp-tRNA synthetase. Following trypsin digestion in the presence of mercaptoethanol, a 4 nmol sample was applied to a 4.6 × 250 mm Brownlee Aquapore RP-300 column. The column was eluted at 1.0 ml/min with the indicated water-methanol gradient in the presence of 0.1% trifluoroacetic acid, room temperature.

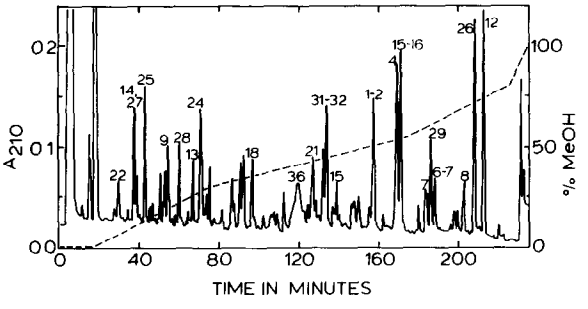


Fig.2. Peptide map of Trp-tRNA synthetase, alkylated post-digestion. A 6 nmol sample was treated exactly as in fig.1, except that it was exposed to 1 mM dithiothreitol followed by 4 mM iodoacetamide before the chromatographic separation of peptides. Newly appearing peaks include the Cys-containing peptides 4, 7, 6–7, and 8, all of which elute between 165 and 205 min.

selected peptides react with the column or are strongly retained by the column. Perhaps more likely is the possibility that large polymers are formed through the random formation of disulfide bonds, particularly since peptide 4 contains two sulfhydryl groups. Polymers formed by such a mechanism would be retained on the column for long periods of time.

These experiments may have important ramifications for sulfhydryl group labeling experiments in other enzyme systems, since those Cys-containing peptides that are not labeled are likely to be unobservable by reversed-phase HPLC.

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